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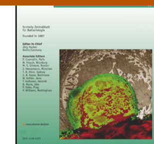

Transcriptional adaptations during long-term persistence of *Staphylococcus aureus* in the airways of a cystic fibrosis patient

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ABSTRACT

The lungs of Cystic fibrosis (CF) patients are often colonized and/or infected by *Staphylococcus aureus* for years, mostly by one predominant clone. For long-term survival in this environment, *S. aureus* needs to adapt during its interactions with host factors, antibiotics, and other pathogens. Here, we study long-term transcriptional as well as genomic adaptations of an isogenic pair of *S. aureus* isolates from a single patient using RNA sequencing (RNA-Seq) and whole genome sequencing (WGS). Mimicking in vivo conditions, we cultivated the *S. aureus* isolates using artificial sputum medium before harvesting RNA for subsequent analysis. We confirmed our RNA-Seq data using quantitative real-time (qRT)-PCR and additionally investigated intermediate isolates from the same patient representing in total 13.2 years of persistence in the CF airways. Comparative RNA-Seq analysis of the first and the last ("late") isolate revealed significant differences in the late isolate after 13.2 years of persistence. Of the 2545 genes expressed in both isolates that were cultivated aerobically, 256 genes were up- and 161 were down-regulated with a minimum 2-fold change (2f). Focusing on 25 highly (≥8f) up- ($n = 9$) or down- ($n = 16$) regulated genes, we identified several genes encoding for virulence factors involved in immune evasion, bacterial spread or secretion (e.g. *spa*, *sak*, and *esxA*). Moreover, these genes displayed similar expression trends under aerobic, microaerophilic and anaerobic conditions. Further qRT-PCR-experiments of highly up- or down-regulated genes within intermediate *S. aureus* isolates resulted in different gene expression patterns over the years. Using sequencing analysis of the differently expressed genes and their upstream regions in the late *S. aureus* isolate resulted in only few genomic alterations. Comparative transcriptomic analysis revealed adaptive changes affecting mainly genes involved in host-pathogen interaction. Although the underlying mechanisms were not known, our results suggest adaptive processes beyond genomic mutations triggered by local factors rather than by activation of global regulators.

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Introduction

Cystic fibrosis (CF) is the most common autosomal recessive genetic disorder in the Caucasian population (O'Sullivan and Freedman, 2009). The malfunction of the CF transmembrane conductance regulator (CFTR), a chloride channel of the epithelial cells in the lung and digestive system in particular, results in thickening of mucus and digestive fluids thereby leading to a strongly impaired mucociliary clearance in the lung (Fahy and Dickey, 2010). As a result, CF patients suffer from chronic airway inflammation and recurrent bacterial infections resulting in progressive pulmonary insufficiency (Konstan et al., 2009). *Staphylococcus aureus* is the most prevalent respiratory pathogen in CF children and adolescents and is one of the first pathogens that can be isolated from CF airways (Cystic Fibrosis Foundation Patient Registry, 2011; Kahl, 2010; Viviani et al., 2012). In adult CF patients, *Pseudomonas aeruginosa* is more common and often replaces *S. aureus*, though the prevalence of *S. aureus* in adults still remains at about 40–60% (Cystic Fibrosis Foundation Patient Registry, 2011; Kahl, 2010). The same clone of *S. aureus* persists in many patients for months or even years (Branger et al., 1996; Kahl et al., 2003b; Renders et al., 1997).

While the natural habitat of *S. aureus* are the anterior nares in the healthy human population (Nouwen et al., 2004), the thick and sticky mucus in the lungs of CF patients presents a new niche.

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Moreover, CF airway disease is characterized by the abundance of polymorphonuclear neutrophils (Cohen and Prince, 2012) and by the frequent presence of co-colonizing species such as *P. aeruginosa* or *Haemophilus influenza*, which compete with *S. aureus* for nutrients. In addition, bacterial pulmonary infections are frequently treated with antibiotics and various ecological niches (e.g. aerated and non-aerated areas) occur in the CF lung environment (Schobert and Tielen, 2010; Tunney et al., 2008; Worlitzsch et al., 2002) that direct the selection of differently adapted variants, as shown for *P. aeruginosa* (Hoboth et al., 2009; Hogardt et al., 2007). Therefore, to survive in this hostile niche *S. aureus* has to adapt to changing selective pressures during long-term persistence. Some adaptive mechanisms have been discovered which account for the diversity observed in the CF *S. aureus* population, such as phage insertions and excisions (Goerke et al., 2004), changes in the protein A gene repeat region (Kahl et al., 2005) and emergence of small-colony variants (SCVs) (Besier et al., 2007; Kahl et al., 1998; Wolter et al., 2013). The SCVs exhibit a smaller colony size, less pigmentation and less hemolysis on Columbia blood agar plates compared to wild-type *S. aureus* strains and are thought to be associated with long-term adaptation to the CF airways (Kahl, 2013; Kahl et al., 2003a,b; Proctor et al., 2006) showing increased antibiotic resistance and the ability to persist intracellularly, thereby protected from the host immune system and antibiotics (Besier et al., 2007; Kahl et al., 1998, 2000).

Whole-genome sequencing has been used to study the adaptation of *S. aureus* to the human host in sequential isolates. Mwangi et al. (2007) detected a stepwise adaptation of *S. aureus* to the host during prolonged acute infection leading to vancomycin resistance, yielding 35 single nucleotide polymorphisms (SNP) in 31 loci in the final of several sequential isolates in three months. Gao et al. (2010) analyzed two methicillin-resistant *S. aureus* isolates, a normal and a SCV phenotype, recovered from a patient 3.8 months apart. Using whole-genome sequencing and microarray transcriptional analysis, the authors discovered two novel point mutations in key staphylococcal genes and global transcriptional changes in the SCV strain promoting persistent *S. aureus* infection (Gao et al., 2010). McAdam et al. (2011) sequenced three sequential *S. aureus* isolates, which were obtained from the sputum of one individual CF patient after 11 and 26 months. The authors identified variation in phage content, genetic polymorphisms in genes associated with antibiotic resistance (e.g. *fusA*) and global regulation of virulence (e.g. *codY, sigB*) (McAdam et al., 2011).

Whereas genomic and phenotypic changes have been investigated, the transcriptional changes as part of long-term adaptive processes of *S. aureus* in the CF lung are not yet well understood. To get deeper insights into the changes of transcription of genes, we employed next-generation RNA-Sequencing (RNA-Seq) on rRNA-depleted total RNA as screening method and quantitative realtime PCR (qRT-PCR) for confirmation of transcriptional changes of an isogenic pair of sequential *S. aureus* isolates recovered from the airways of an individual CF patient 13.2 years apart. From the same pair of isolates, we determined the complete genome sequence, which served not only as optimal reference sequence for RNA-Seq data analysis but also helped to elucidate potential genomic changes in differentially regulated genes. To mimic the CF lung habitat, we cultivated the *S. aureus* isolates in an artificial sputum medium (ASM) (Sriramulu et al., 2005). Based on our RNA-Seq data, we selected a number of genes, which were highly differentially expressed in the last ("late") *S. aureus* isolate and confirmed transcriptional changes by qRT-PCR. Furthermore, using five additional intermittent sequential *S. aureus* isolates recovered from the same patient, we examined some of these genes by qRT-PCR to detect specific expression patterns over the course of time. To determine the influence of variable oxygen saturations on the gene expression, we selected interesting candidate genes that were differently expressed in the late isolate under aerobic conditions, and tested their expression in both isolates under microaerophilic and anaerobic conditions.

Materials and methods

Bacteria, culture conditions and RNA preparation

Seven sequential isogenic *S. aureus* isolates, which were recovered from the airways of a male CF patient (born in 1993) from 1995 to 2008 (patient 20 in Hirschhausen et al. (2013)), were selected for further analysis (Supplementary data: Table S1). The early and intermediate *S. aureus* isolates were isolated from the upper airways (throat or nasal swabs) and the late isolate from a sputum sample of the lower airways. Co-colonization or infection with *P. aeruginosa* was not detected in the patient during the study period. All isolates were penicillin resistant, but methicillin sensitive and exhibited a normal phenotype regarding colony size. All isolates belonged to the MLST sequence type (ST) 30 and *spa* type t012 or the related *spa* type t021 (deletion of a single repeat changed the *spa* type t012 to t021) and shared an identical PFGE pattern (Supplementary data: Table S1). All isolates were stored at −80 °C until analysis and freshly cultured for all experiments.

The *S. aureus* isolates were cultivated in ASM (Sriramulu et al., 2005) containing 25 mg/ml albumin (Serva Electrophoresis GmbH, Heidelberg, Germany), 5 mg/ml mucin from porcine stomach (Sigma, Sigma-Aldrich Chemie GmbH, München, Germany), 2.7 mg/ml DNA from fish sperm (Serva), 0.8 mg/ml glucose, 0.25 mg/ml amino acids (Sigma), 69 mM NaCl, 3 mM $CaCl_2$, 20 mM HEPES (Sigma), 0.5 mM urea, 1.0% FBS (Fiebrig Nährstofftechnik, Idstein-Niederauroff, Germany), and 0.5% egg yolk emulsion (Sigma), pH 6.9. Before RNA extraction, we performed growth curve experiments to determine the time point of the logarithmic growth phase for RNA extraction. Due to the specific characteristics of the ASM medium to mimic the in vivo conditions, we observed a time span between 4 and 16 h as logarithmic growth phase (data not shown). The isolates were grown aerobically, microaerophilic (AnaerocultA, Merck GmbH, Darmstadt, Germany) or anaerobic (AnaerocultC, Merck) at 37 °C and 100 rpm, after dilution of an overnight culture to a starting optical density of 0.1. After 6 h of growth, total RNA was purified harvesting the bacteria and twice the volume of the culture volume of RNAprotect Bacteria Reagent (Qiagen GmbH, Hilden, Germany) was used for in vivo stabilization of total RNA. Subsequently, total RNA was extracted with the RNeasy Kit (Qiagen) using the bacterial lysis protocol recommended by the manufacturer in combination with a mechanical disruption of bacteria via glass beads (≤106 μm diameter, Sigma). Specifically, for cell lysis the bacteria were incubated in TE (10 mM TrisCl, 1 mM EDTA, pH 7.0) containing 83 μg/ml lysostaphin (Sigma) and 33 mg/ml Proteinase K (Carl Roth GmbH, Karlsruhe, Germany) for 40 min at 37 °C. In addition, to yield higher amounts of total RNA and reduce genomic DNA contaminations, gDNA eliminator spin columns and RLT plus puffer (Qiagen) were used during the RNA extraction protocol. Moreover, total RNA was DNase I digested twice (RNase-Free DNase I, Qiagen, 2× or 4× final concentration, as recommended by the manufacturer) in reaction volumes of 50 or 100 μl, incubated for 20 minutes at room temperature and cleaned up by the RNA clean up protocol. Total RNA was checked for DNA contamination via PCR and further quality controlled using the Agilent 2100 bioanalyzer according to the Agilent RNA 6000 nano protocol (Agilent Technologies, Inc., Santa Clara, CA, USA). Only RNA samples of high quality (RIN value ≥8.0) were used for cDNA library construction and/or reverse transcription prior to qRT-PCR. Total RNA was stored at −20 °C. During RNA preparation, RNA samples (technical replicates) were pooled before DNase

I digestion. RNA-Seq and qRT-PCR experiments were carried out on different RNA samples (technical replicates) to ensure an independent data analysis for each approach.

Whole genome sequencing of the early and late S. aureus isolate and de novo assembly

For whole genome shotgun sequencing of the early (F343795) and the late (CF74808) *S. aureus* isolate, sequencing libraries were prepared using the Nextera XT chemistry (Illumina) for a 100 bp paired-end sequencing run on an Illumina HiScanSQ sequencer in accordance to the manufactures' recommendations (Illumina). To achieve a single chromosomal sequence (one contig) WGS was additionally performed on a PacBio *RSII* (Pacific Biosciences, Menlo Park, CA, USA). Therefore, SMRTbell™ template libraries were prepared according to the manufactures' instructions (Pacific Biosciences), following the Procedure & Checklist for 10 kb Template Preparation and Sequencing. Briefly, for preparation of 10 kb libraries ~5 μg genomic DNA was end-repaired and ligated to hairpin adapters applying components from the DNA/Polymerase Binding Kit 2.0. Reactions were carried out according to the manufacturer's instructions. SMRTbell™ templates were Exonuclease treated for removal of incompletely formed reaction products. Conditions for annealing of sequencing primers and binding of polymerase to purified SMRTbell™ templates were assessed with the Calculator in RS Remote (Pacific Biosciences). SMRT sequencing was carried out taking one 180-min movie for each SMRT cell. In total five SMRT cells were run for the early and four SMRT cells for the late isolate. De novo genome assemblies for both strains were performed using the "RS_HGAP_Assembly.2" protocol included in SMRTPortal version 2.1.1 using standard parameters. Afterwards, insertion errors were eliminated from all replicons by a mapping of paired end Illumina HiScanSQ reads with BWA (Li and Durbin, 2009) and subsequent variant calling as well as consensus correction within CLC Genomics Workbench 6.5 (CLC bio, Aarhus, Denmark). Thus, a highly curated consensus sequence was extracted, which finally was handed over to the "RS_BridgeMapper.1" protocol within SMRTPortal 2.1.1.

Prior to RNA-seq data analysis, the two chromosomal sequences were automatically annotated using the online xBASE bacterial genome annotation service with default parameters (Chaudhuri et al., 2008).

cDNA library construction and cDNA sequencing

cDNA libraries for the early and late *S. aureus* isolate were constructed by Vertis Biotechnology AG (http://www.vertis-biotech.com/). Briefly, primary bacterial transcripts (most mRNAs and sRNAs) which carry a 5′ triphosphate (5′PPP) were enriched from total RNA samples using Terminator 5′-phosphate dependent exonuclease (TEX, Epicentre, Madison, WI, USA) (Sharma et al., 2010). After fragmentation of RNA by ultrasound treatment, the RNA probes were tobacco acid pyrophosphatase (TAP, Epicentre) treated to remove 5′PPP from primary transcripts. 3′-ends of RNA were poly(*A*) tailed using poly(*A*) polymerase and a synthetic RNA adapter was ligated to 5′*P*-ends of RNA fragments. First-strand cDNA synthesis was applied using an oligo (d*T*)-adapter primer and M-MLV *H*-reverse transcriptase. The synthesized cDNAs were PCR-amplified to about 20–30 ng/μl using a high fidelity DNA polymerase and primers designed for TruSeq sequencing (Illumina Inc., San Diego CA, USA). Specific barcode sequences (ATCACG or CGATGT) were attached to the 5′ends of the cDNAs. After synthesis, the cDNA was purified by the Agencourt AMPure XP kit (Beckman Coulter Genomics, Danvers, MA, USA) and analyzed on a Shimadzu MultiNA microship electrophoresis system (Shimadzu Deutschland GmbH, Duisburg, Germany). For sequencing on an Illumina HiScanSQ sequencer (Illumina), cDNA libraries were pooled using equimolar amounts of the different samples and 100 bp single reads were generated.

Read mapping and analysis of RNA sequencing data for transcriptomic analysis

For comparative transcriptome analysis, we used the CLCbio Genomics Workbench software version 7.04 (CLCbio) using default parameters. As reference sequences for mapping of the sequencing reads the chromosome of *S. aureus* F343795 (early isolate) was selected (ENA study accession no PRJEB5528). Reads that mapped on rRNA encoding genes were removed as an in-silico rRNA depletion step using the READemption tool (Förstner et al., 2014) and the resulting reads were subsequently used for the final analysis. After mapping of these reads on the reference genome, we used the raw read counts for each gene for normalization and subsequent determination of transcriptional changes between the two *S. aureus* isolates (fold change analysis). The fold change (f) was calculated using the early isolate as a reference and the results were stratified into differently expressed genes in the late isolate with ≥2f, ≥4f, or ≥8f and into genes that were either not expressed in both isolates or not present on the genome level in both isolates. For exclusion of low abundant transcripts, we used a threshold of less than 3 reads per gene. For further analysis, we focused on subsets of genes that were up- or down-regulated in the late isolate with a ≥8f.

Quantitative real-time PCR (qRT-PCR) from RNA

To confirm RNA-Seq data, differently expressed genes in the late *S. aureus* isolate exhibiting ≥8f and if applicable further related genes were selected for qRT-PCR. Primers were designed for candidate genes and the housekeeping gene *hup* (DNA-binding protein HU, locus tag F343795_1414), which was used as reference gene for normalization, as it showed comparable low fold changes in comparison to other housekeeping genes suggested by Derzelle et al. (2009). For primer design, the software Primer3 was used (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primer sequences (Supplementary data: Table S2) were designed based on the gene sequences of *S. aureus* F343795 (early isolate) and checked for gene specificity by NCBI-BLAST search in the genome. In a two-step qRT-PCR, total RNA was synthesized to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). The Kit includes a DNase digestion step to eliminate remaining genomic DNA. For cDNA synthesis, 0.56 μg of total RNA were used in a final reaction volume of 80 μl following the reverse transcription procedure: reverse transcription 42 °C for 15 min and enzyme inactivation 95 °C for 3 min. Negative controls (without reverse transcriptase) were checked for genomic DNA contaminations. We used the QuantiTect SYBR Green PCR Kit (Qiagen) for the second step (the dye ROX was used for fluorescence normalization) and performed the reactions in a StepOnePlus real-time PCR machine (Applied Biosystems, Foster City, CA, USA) under following cycling conditions: one initial PCR activation step at 95 °C for 15 min followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 53 °C for 30 s, and elongation at 72 °C for 30 s. For specificity testing, a melting curve analysis was added. As template, either 0.8 μl of cDNA or water (no-template control) was used for qRT-PCR reactions (in duplicates) in a total volume of 15 μl containing both primers in a final concentration of 300 nM. A relative quantification (RQ) value was calculated using default parameters of the StepOne Software version 2.1 (Applied Biosystems) for each target gene using the early *S. aureus* isolate as a reference and *hup* for normalization.

Analysis of genomic alterations within candidate genes and their upstream promoter regions

To analyze candidate genes (≥8f, RNA-Seq) or operon genes and their intergenic upstream regions (likely promoter regions) for genomic alterations in the late isolate, we compared the genome sequences of the early and late *S. aureus* isolates per NCBI nucleotide BLAST search to identify point mutations, insertions or deletions in these selected genomic regions.

Results

Genome sequencing of early and late S. aureus isolates

Prior to RNA-Seq, we determined the genome sequences of the early (F343795) and the late (CF74808) *S. aureus* isolates. To accomplish complete closed genome sequences, we applied the Pacific Bioscience technology and included additional Illumina reads that finally assembled in one bacterial chromosome, which was trimmed, circularized and adjusted to *dnaA* as first gene, for both isolates. Herewith, a consensus accuracy of QV 60 (99.9999%) for both chromosomes was confirmed. Additionally, for the early isolate a 30 kb extra-chromosomal element, i.e. a plasmid, was recorded and circularized with a consensus accuracy of 100%. Automated annotation of the chromosome resulted in 2743 CDS and 79 RNAs (19 rRNAs, 59 tRNAs, and 1 miscRNA). The chromosomal sequence of the early isolate served as reference sequence for RNA-Seq data analysis; the plasmid was not used as reference sequence since it was missing in the late isolate. The comparison of both sequences helped to elucidate potential genomic changes in the differentially regulated genes identified by RNA-Seq.

The curated whole genome sequences have been deposited at ENA (study accession nos. PRJEB5528 and PRJEB5533).

Comparative transcriptomic analysis between two sequential S. aureus isolates

To study transcriptional changes between the early and the late *S. aureus* isolate, which were recovered from the airways of an individual CF patient 13.2 years apart, both isolates were cultivated using ASM and the transcriptome was characterized using RNA-Seq. In total, 15.362809 and 12.752567 cDNA reads were used for subsequent analysis from the early and the late isolate, respectively. After removal of all reads that mapped on rRNAs, 1.981034 reads (12.9%) of the early and 2.654981 reads (20.8%) of the late isolate were mapped onto the reference sequence (the chromosomal sequence of the early isolate F343795) and filtered for 2803 features (2743 CDS, 59 tRNAs, and 1 miscRNA; the 19 rRNAs were excluded). After normalization, based on raw read counts and subsequent comparative analysis, in total 256 (10.1%) of the 2545 genes expressed in both isolates were up-regulated (≥2f) in the late isolate, and 161 (6.3%) genes were down-regulated (≥2f). One-hundred thirty-eight (4.9%) genes were excluded due to low abundance of transcripts (less than 3 reads per gene present). We further stratified the fold change data using further thresholds ≥4f and ≥8f (Fig. 1, Tables 1 and 2, Supplementary data: Table S3). Due to their absence of mapped cDNA reads in at least one of the two isolates, 120 genes displayed no fold change (Supplementary data: Table S3). Genes, that were up- or down-regulated in the late isolate with ≥8f (Tables 1 and 2), were selected for further analysis and subjected to qRT-PCR analysis for validation.

Up-regulated genes in the late isolate with ≥8-fold change

Of the 256 up-regulated genes, 9 genes were up-regulated in the late *S. aureus* isolate with ≥8f (Table 1). In all genes that were

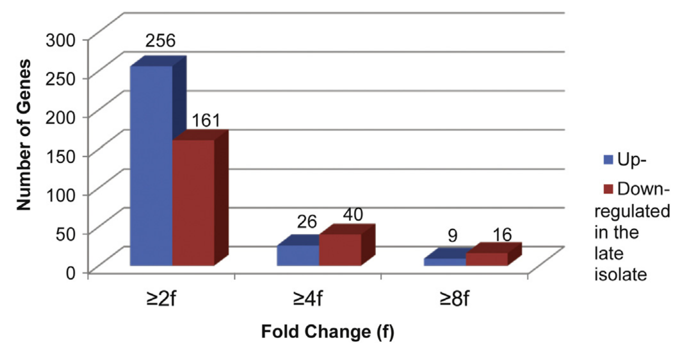

Fig. 1. Comparative transcriptomic analysis of the late *S. aureus* isolate versus the early isolate. The histogram displays the number of genes with increased or decreased transcription as fold change values of the late isolate in comparison to the early isolate. In total, 2803 features were analyzed (2743 CDS, 59 tRNA and 1miscRNA), of these 2545 were expressed in both isolate with at least three reads per gene.

chosen for qRT-PCR confirmation, qRT-PCR results corroborated with RNA-Seq data (Table 1). The genes *esxA*, *esaA*, *essA*, and *essC* were members of the Ess pathway (ESAT-6-like secretion system), which comprises a cluster of at least nine genes *esxA*, *esaA*, *essA*, *esaB*, *essB*, *essC*, *esaC*, *esxB*, and *esaD* (Anderson et al., 2011) (Table 3, Supplementary data: Fig. S1). We therefore also characterized the other genes of this pathway irrespective of their fold change values (Table 3). Interestingly, with the exception of *esaB*, which was present in reads of the late isolate only (Supplementary data: Table S3), none of the other Ess pathway genes (*esaC*, *esxB*, *esaE*, *esaF* and *esaD*), that can be found in *S. aureus* Newman (NC_009641.1) could be detected in the genome sequences of the two isolates. The majority of the other up-regulated genes did not belong to an operon, but represented important virulence or immune evasion genes (*aur*, *sak*, *spa*) or were coding for hypothetical proteins only (Table 1).

Down-regulated genes in the late isolate with ≥8-fold change

Sixteen genes were down-regulated in the late *S. aureus* isolate with ≥8f (Table 2). Among these genes, three (*kdpC*, *kdpB* and *kdpA*) belonged to the *kdpFABC* operon (Table 4, Supplementary data: Fig. S2), encoding for a potassium (K^+)-transporting ATPase (Xue et al., 2011). No fold change could be calculated for the gene *kdpF* (locus tag F343795_2182a, nucleotide position c2242,603–2242,493) since cDNA reads of *kdpF* were only present in the early isolate (Supplementary data: Table S3). However, we subsequently analyzed all four *kdp* genes, *kdpFABC*, per qRT-PCR. The data confirmed that all four genes were down-regulated in the late isolate with comparable ratios (Tables 2 and 4). Moreover, two genes (locus tags F343795_1914 and F343795_2110, Table 2) encoding an amidase and the toxic shock syndrome toxin-1, displaying down-regulation in the late isolate with high fold changes in RNA-Seq data. However, as both genes were not completely found in the genome sequence of the late isolate, we considered the detected reads in the late isolate as unspecifically mapped reads and excluded both genes from further analysis (Table 2).

Genomic alterations within candidate genes and their upstream promoter regions

To determine whether changes in gene expression were due to genomic alterations either within the respective gene or upstream in the promoter region of the gene or operon, we used the whole genome sequences of both isolates and analyzed the affected genes or operons and their upstream regions (intergenic regions). Besides the change of the spa type that became obvious already during

Table 1
Up-regulated genes ($n = 9$) in the late *S. aureus* isolate with ≥8f in RNA-Seq.

Locus tag (gene)	Target range[a] (size)	Fold change	Gene product	qRT-PCR results (log2 RQ)
F343795_0061 (*spa*)	c71,987–70,425 (1563 bp)	61.3	IgG-binding protein A precursor	Confirmed RNA-Seq (8.12)
F343795_0228 (*esxA*)	271,337–271,630 (294 bp)	10.1	EsxA of the Ess secretion pathway	Confirmed RNA-Seq (5.73)
F343795_0229 (*esaA*)	271,713–274,742 (3330 bp)	14.0	EsaA the Ess secretion pathway	Confirmed RNA-Seq (3.41)
F343795_0230 (*essA*)	274,742–275,200 (449 bp)	8.9	EssA the Ess secretion pathway	Confirmed RNA-Seq (4.25)
F343795_0233 (*essC*)	276,831–281,231 (4401 bp)	12.7	EssC the Ess secretion pathway	Confirmed RNA-Seq (4.40)
F343795_2045 (*sak*)	c2121,492–2121,001 (492 bp)	9.9	Staphylokinase precursor	Confirmed RNA-Seq (2.18)
F343795_2121	2180,739–2181,106 (368 bp)	12.8	Hypothetical protein	Not determined
F343795_2740 (*aur*)	c2817,268–2815,739 (1530 bp)	11.3	Zinc metalloproteinase aureolysin	Confirmed RNA-Seq (2.50)
F343795_2807	c2896,825–2896,151 (675 bp)	8.7	Putative exported protein	Not determined

[a] The prefix c marks the complementary orientation of the gene.

Table 2
Down-regulated genes ($n = 16$) in the late *S. aureus* isolate with ≥8f in RNA-Seq.

Locus tag (gene)	Target range[a] (size)	Fold change	Gene product	qRT-PCR results (log2 RQ)
F343795_0466	504,377–504,501 (125 bp)	8.7	tRNA-Gly	Not determined
F343795_0539	590,038–590,357 (320 bp)	10.5	Hypothetical protein	Not determined
F343795_0540 (*vraX*)	c590,558–590,775 (218 bp)	25.1	VraX	Confirmed RNA-Seq (−5.57)
F343795_1086	1152,930–1153,114 (185 bp)	9.3	Anti protein	Not determined
F343795_1649	1748,685–1749,190 (506 bp)	12.5	Hypothetical protein	Not determined
F343795_1914[b]	c2009,644–2008,199 (1446 bp)	175.4	Amidase	N/A
F343795_1982 (*vraS*)	2055,895–2056,988 (1094 bp)	9.1	Histidine kinase sensor	Not determined
F343795_2110[b] (*tst*)	2172,703–2173,407 (705 bp)	457.7	Toxic shock syndrome toxin-1	N/A
F343795_2180 (*kdpC*)	c2238,685–2238,167 (519 bp)	44.9	Potassium-transporting ATPase subunit C	Confirmed RNA-Seq (−4.14)
F343795_2181 (*kdpB*)	c2240,783–2238,747 (2037 bp)	59.6	Potassium-transporting ATPase subunit B	Confirmed RNA-Seq (−4.32)
F343795_2182 (*kdpA*)	c2242,445–2240,793 (1653 bp)	59.8	Potassium-transporting ATPase subunit A	Confirmed RNA-Seq (−5.27)
F343795_2317	2385,749–2386,224 (476 bp)	11.6	Hypothetical protein	Not determined
F343795_2472	2524,542–2524,997 (456 bp)	20.5	Putative membrane protein	Not determined
F343795_2544	c2602,016–2601,924 (93 bp)	21.1	Hypothetical protein	Not determined
F343795_2608	2673,273–2674,063 (791 bp)	9.5	MerR family regulatory protein	Confirmed RNA-Seq (−2.62)
F343795_2660	2728,935–2729,135 (201 bp)	17.8	Putative exported protein	Not determined

N/A, not applicable.
[a] The prefix c marks the complementary orientation of the gene.
[b] These genes are either completely absent or at least partially (below a DNA sequence similarity threshold of 90% and an overlap of <99%) missing in the genome of the late isolate. We therefore rated these results as unspecific read mappings and excluded these genes from further analysis.

initial spa typing (deletion of a single 24 bp repeat), we found only few genomic alterations (Supplementary data: Table S4). In the remaining investigated genes, the further detected point mutations were not located in promotor regions up to 50 bp upstream the transcription start site (Supplementary data: Table S4).

Gene-expression in intermediate S. aureus isolates

A subset of the genes that were up- or down-regulated in the late isolate (≥8f) were further analyzed in five isogenic intermediate *S. aureus* isolates of this patient (Supplementary data: Table S1)

Table 3
Expression values of the Ess pathway genes of *S. aureus*.

Locus tag (gene)	Fold change (up-regulated)	Gene product	Reference(s)
F343795-0228 (*esxA*)	10.1	EsxA secreted by the Ess system, virulence factor	Anderson et al. (2011), Burts et al. (2005)
F343795-0229 (*esaA*)	14.0	EsaA membrane protein	Anderson et al. (2011), Burts et al. (2005)
F343795-0230 (*essA*)	8.9	EssA membrane protein, essential for the transport of EsxA, EsxB and EsaC across the bacterial envelope	Anderson et al. (2011), Burts et al. (2005)
F343795-0231 (*esaB*)	–[a]	EsaB cytoplasmic protein, negative regulator of EsaC	Anderson et al. (2011), Burts et al. (2008)
F343795-0232 (*essB*)	–[a]	EssB membrane protein, essential for the transport of EsxA, EsxB and EsaC across the bacterial envelope	Anderson et al. (2011), Burts et al. (2005), Chen et al. (2012)
F343795-0233 (*essC*)	12.7	EssC membrane protein, essential for the transport of EsxA, EsxB and EsaC across the bacterial envelope	Anderson et al. (2011), Burts et al. (2005)
esaC[b] or *esxC*	N/A	EsaC secreted by the Ess system, virulence factor	Anderson et al. (2011), Burts et al. (2008)
esxB[b]	N/A	EsxB secreted by the Ess system, virulence factor	Anderson et al. (2011), Burts et al. (2008, p. 200, 2005)
esaE[b]	N/A	EsaE function unknown	Anderson et al. (2011)
esaF[b] or *esxD*	N/A	EsaF or EsxD secreted by the Ess system	Anderson et al. (2013), Anderson et al. (2011)
esaD[b] or *essD*	N/A	EsaD, membrane protein, required for EsxA secretion	Anderson et al. (2011)

N/A, not applicable.
[a] Not expressed in the early isolate (F343795).
[b] These genes are missing in *S. aureus* F343795 and CF74808.

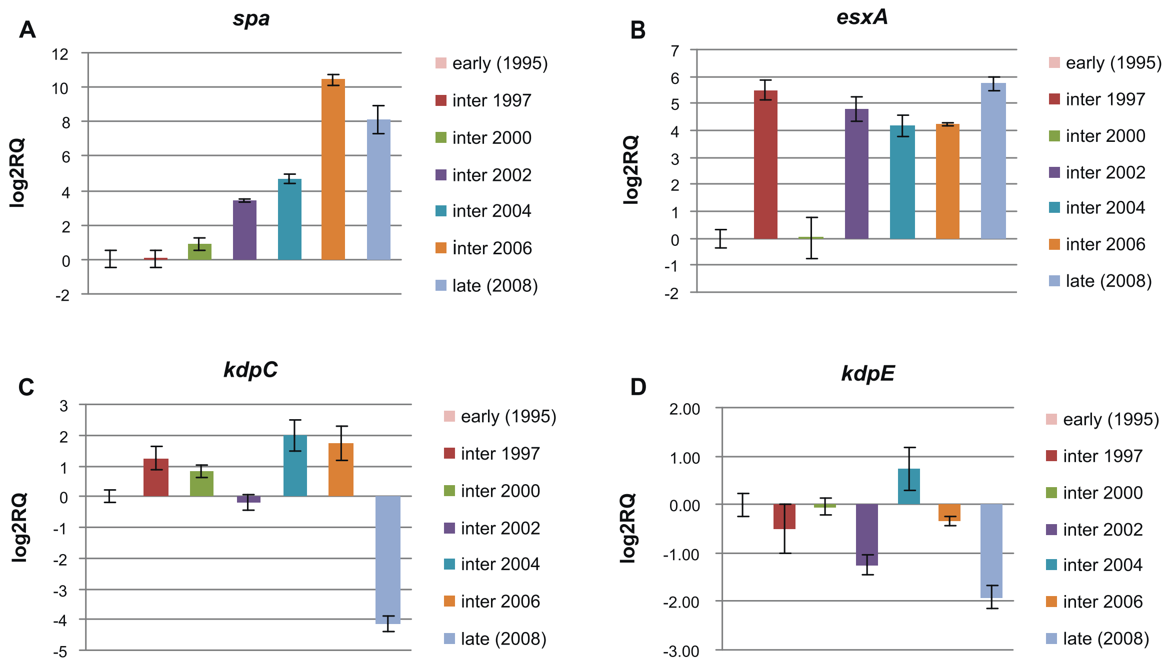

Fig. 2. Gene expression patterns in intermediate *S. aureus* isolates. Three candidate genes *spa* (A), *esxA* (B), *kdpC* (C) that were highly differentially expressed in the late isolate (≥8f) and *kdpE* (D) were selected and investigated by qRT-PCR in five additional (intermediate) isolates to determine gene expression patterns over the course of long-term persistence of *S. aureus* in the CF airways. The early isolate was used as a reference to calculate the relative quantification (RQ) value for each target gene.

Table 4
Expression values of the Kdp operon of *S. aureus* (Xue et al., 2011).

Locus tag (gene)	Fold change (down-regulated)	Gene product
F343795_2180 (*kdpC*)	44.9	Potassium-transporting ATPase subunit C
F343795_2181 (*kdpB*)	59.6	Potassium-transporting ATPase subunit B
F343795_2182 (*kdpA*)	58.9	Potassium-transporting ATPase subunit A
F343795_2182a (*kdpF*)	–[a]	Potassium-transporting ATPase subunit F
F343795_2183 (*kdpD*)	2.7	Sensor kinase protein
F343795_2184 (*kdpE*)	3.1	Response regulator protein

[a] Gene is present with an identical sequence in both isolates but not expressed in the late isolate (CF74808).

using qRT-PCR to determine gene expression patterns over the course of long-term persistence using the early isolate (from 1995) as a reference. Interestingly, we observed different patterns of gene expression for *S. aureus* during long-term adaptation in the CF airways. For *spa*, we noticed an increase in gene expression in the course of time. In the early years, 1997 and 2000, *spa* was expressed at relatively low ratios. Starting from 2002, a clear increase in gene expression (up-regulation) was observed, reaching its peak in 2006 (Fig. 2A). The Ess pathway gene *esxA* was highly expressed at comparable gene expression ratios over the years, except for 2000 when a low ratio was monitored (Fig. 2B). The *kdp* operon gene *kdpC* was expressed lowly in nearly all intermediate isolates, but shifted to down-regulation in the late isolate in 2008 (Fig. 2C). For *kdpE* a similar pattern as for *kdpC* could be observed with very low expressions in intermediate isolates and a trend to down-regulation in the late isolate (Fig. 2D).

Gene expression of candidate genes under different pO_2

Since various ecological niches exist in the CF lung, such as oxygen-limited and anaerobic environments (Schobert and Tielen, 2010; Tunney et al., 2008; Worlitzsch et al., 2002), we wondered whether the genes that were highly expressed in the late isolate (≥8f) under aerobic conditions, would also show similar gene expression patterns under microaerophilic and anaerobic conditions. Analysis of the gene expression of six genes (*esxA*, *aur*, *kdpC*, *kdpE*, *sak*, and *spa*) in the early and late *S. aureus* isolate under aerobic, microaerophilic and anaerobic conditions resulted in similar expression tendencies in nearly all genes (the only exception was *kdpE* under anaerobic conditions) under all three pO_2 conditions irrespective whether the genes were up- or down-regulated (Fig. 3A–C). Whereas *spa* transcription rates increased in the course of time (Fig. 2A) and in the comparative analysis under all pO_2 conditions tested (Fig. 3A–C), in relation to *hup* alone *spa* is down-regulated under oxygen-limited and even more under anaerobic conditions (Table S5) corroborating previous findings by Pragman et al. (2004).

Discussion

Based on RNA-Seq data that were mapped on the originating chromosomal sequences, we were able to determine transcriptional changes between sequential *S. aureus* isolates during 13.2 years of long-term persistence in a CF patient. In general, we observed that a much higher number of genes were up- than down-regulated in the late isolate with ≥2f. An explanation for this considerable difference might be the selective pressure that leads to a general activation of many genes in order to facilitate survival. For deeper analyses, we selected a smaller set of candidate genes that were highly differentially expressed in the late isolate (≥8f). After validation of the RNA-Seq data using qRT-PCR, focusing on 25 genes that were highly up- (Table 1) or down-regulated (Table 2), we were able to identify several genes encoding for virulence factors that have an impact on the host-pathogen interaction, e.g., by facilitating immune evasion during persistence in the lung.

A major factor in the evasion of the host immune system, the gene encoding staphylococcal protein A (Spa), was highly upregulated in the late isolate. Spa allows for IgG by binding of Fc fragments and enables the microorganism to evade host immune

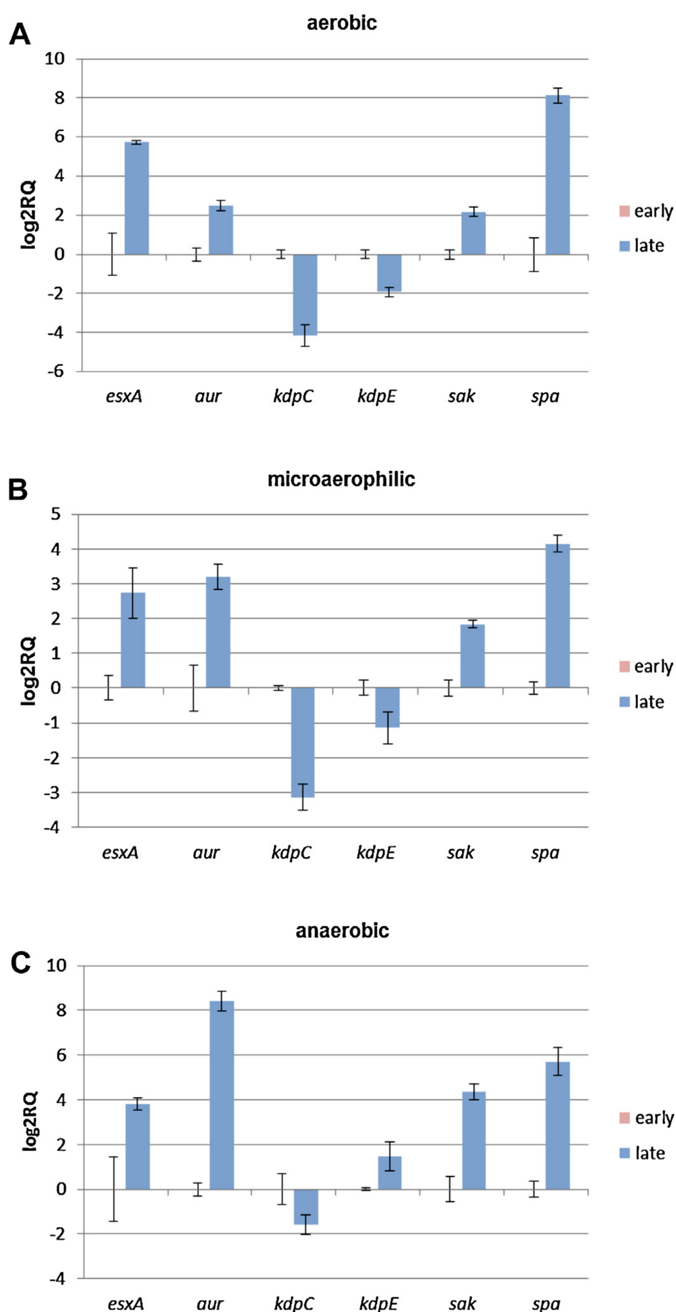

Fig. 3. Gene expression of candidate genes under different pO_2. Candidate genes that were highly differently expressed in the late isolate under aerobic conditions (≥8f) and *kdpE* were selected and investigated by qRT-PCR under aerobic (A), microaerophilic (B) and anaerobic (C) conditions mimicking aerated, oxygen-limited and non-aerated niches in the CF lung environment. The early isolate was used as a reference to calculate the relative quantification (RQ) value for each target gene.

response to facilitate survival and establish infection. Anchored to the cell surface, Spa binds IgG Fc fragments, blocks their recognition by Fc receptors on neutrophils, and thereby prevents phagocytosis and classical pathway complement fixation (Atkins et al., 2008; Foster, 2005). Whether mutations within the repeat region of *spa* might explain the increased transcription rate of *spa* is not known. However, in a previous study we have already detected genomic changes within *spa* from sequential *S. aureus* isolates that might indicate its influence on long-term persistence in CF patients (Kahl et al., 2005). Here, we detected a new, closely related *spa* type for the first time in the isolate from 2002. Interestingly, a repeat of the variable region was deleted during persistence, which is in line with the observation of Garofalo et al. (2012), who described that during chronic osteomyelitis the number of *spa* repeats decreased in *S. aureus* isolates and that such mutations were associated with a reduced inflammatory response.

Two other genes encoding for staphylokinase (SAK, locus tag F343795_2045) and aureolysin (locus tag F343795_2740) were also among the ≥8f up-regulated genes. Interestingly, both gene products have similar functions as they facilitate the bacterial spread in the extracellular matrix via activation of the fibrinolysis and degradation of extracellular matrix proteins (Beaufort et al., 2008; Bokarewa et al., 2006; Braff et al., 2007; Parry et al., 2000; Stephens and Vaheri, 1993). Moreover, SAK and aureolysin are involved in the evasion of the host immune system by interacting via different mechanisms of the innate and adaptive immune system (Jin et al., 2004; Prokešová et al., 1991; Rooijakkers et al., 2005). Degradation of antimicrobial peptides like cathelicidin (Sieprawska-Lupa et al., 2004), which is produced by alveolar macrophages and airway epithelial cells at the sites of infection (Agerberth et al., 1999; Bals and Hiemstra, 2004), and of different components of the complement system (Laarman et al., 2011; Rooijakkers et al., 2005) by proteases is a common strategy to escape host complement responses that has also been demonstrated in other gram-positive bacteria like Group A streptococci (Nizet, 2007). We therefore assume that the detected up-regulation of staphylokinase and aureolysin fosters the survival of *S. aureus* during long-term persistence in the CF-lung.

Besides single genes, we also found several genes of a pathway up-regulated: four genes of the Ess pathway (*esxA*, *esaA*, *essA*, *essC*) were up-regulated in the late isolate. The pathway was initially described in *M. tuberculosis* as a secretion system for two virulence factors (ESAT-6 and CFP-10) (Berthet et al., 1998; Guinn et al., 2004; Sørensen et al., 1995; Stanley et al., 2003), but later homologues of ESAT-6 have also been identified in *S. aureus* (Pallen, 2002). The exact function of this pathway in pathogenicity is still unclear. In our analysis we found only four genes of this pathway. The remaining genes, namely *esaC*, *esxB*, *esaE*, *esaF*, and *esaD*, were missing in the genome sequences of the early and late isolate. Via BLAST, we searched for Ess genes in the 33 complete *S. aureus* genomes that were available in the NCBI database at the time of this investigation (25th January, 2013) using the Ess genes of the Newman strain (NC_009641.1: NWMN_0219–NWMN_0228). Similar to our isolates, the same Ess genes (*esaC*, *esxB*, *esaE*, *esaF* and *esaD*) were missing in twelve of the 33 published genomes (strains MRSA252, MN8, M013, TW20, ST398, LGA251, JKD6008, TCH60, T0131, 71193, HO 5096 0412 and 08BA02176). Anderson et al. (2011), reported a *bursa aurealis* insertion in *esaD* in *S. aureus* strain USA300 that resulted in a reduced secretion of EsxA. However, these missing genes might not be crucial for the pathway function. The remaining proteins of the Ess pathway genes, which are up-regulated in the late isolate, might be sufficient for the transport of EsxA and perhaps for further so far unidentified proteins.

Among the down-regulated genes, the four genes of the *kdp* operon (*kdpFABC*) warrant further attention. Whereas the operon was primarily described as a high-affinity K^+ transporter in several bacteria (Bakker et al., 1987; Garcia-Cuellar et al., 1995; Polarek et al., 1992; Treuner-Lange et al., 1997), it has been shown that in *S. aureus* the transcription of *kdpFABC* is not activated under low external K^+ concentrations by KdpDE, suggesting another function in *S. aureus* (Xue et al., 2011). Indeed, Xue et al. (2011) showed that the transcription of several virulence genes, such as *spa*, *cap*, *hla*, *aur*, *geh*, and *hlgB*, were altered in a *kdpDE S. aureus* mutant, and that KdpE can specifically bind to the promotor regions of the genes, except *hla*, in vitro (Xue et al., 2011). However, Xue et al. also found out that a specific binding of KdpE to *kdpDE* did not influence their transcription; therefore, we cannot infer from our

transcriptional data alone the KdpE activity. We additionally found two synonymous point mutations in *kdpB* and *kdpE* and an insertion of an adenine (A) in *kdpD* (Supplementary data: Table S4); however, as the expression patterns in the intermediate isolates were also different to the patterns of *spa*, we refuted the hypothesis of a linked transcription.

One limitation in our study might be the fact that we used aerobic conditions as a starting point for our analysis though many areas of the CF lung are only poorly aerated. As the characterized genes showed similar expression tendencies under all three pO_2 conditions (aerobic, microaerophilic, anaerobic) irrespective whether they were up- or down-regulated (Fig. 3), we concluded that variation in the pO_2 was not the trigger for the initially detected transcriptional changes between the early and the late isolate under aerobic conditions. Due to our starting point "aerobic conditions", however, all genes that might be highly up- or down-regulated under microaerophilic or anaerobic conditions are excluded. Moreover, in our approach we might have lost additional candidate genes if they were not expressed in both isolates. As it became obvious in the meantime that sequencing with a lower depth and performing more biological replication should be preferred over increased sequencing depth (Rapaport et al., 2013), we see another potential limitation of our analysis as we used only one biological replicate for RNA-Seq with high sequencing depth. However, as we employed qRT-PCR for confirmation of RNA-Seq results, we believe that this technical limitation did not considerably bias our analysis.

In summary, our transcriptome data demonstrated that excessive transcriptional changes occur in *S. aureus* isolates during long-term adaptation and that several genes are highly up-regulated in the late *S. aureus* isolate after 13.2 years of persistence in the CF lung. These genes encode proteins that play a role in immune evasion, inhibit the host adaptive and innate immune system or inactivate bactericidal peptides. Since SAK and aureolysin are also able to manipulate and activate the host fibrinolytic system, bacterial spread might also be an important mechanism enabling the bacteria to colonize new niches in the CF lung. Analyzing the gene expression in intermediate isolates, we detected a clear up-regulation of *spa* in the last six years of persistence that might illustrate the significance of this protein for *S. aureus* especially during later years of persistence in the CF airways. In contrast, we also observed different patterns of gene expression for the other tested genes over the years, which might reflect on the pathogen's side either localized effects or effects of different *S. aureus* regulatory systems on the gene expression. Regarding the host's side, changes in the CF lung micro-environment that occurred prior to the isolation of our isolates might have contributed to the adaptive changes. Therefore, future studies will not only focus on the host side to elucidate factors that might have an impact on the establishment of transcriptional changes during long-term adaptation thereby contributing to the adaptation of *S. aureus* in the CF airways, but also try to determine simultaneously the transcription rate of the pathogen and the host using dual RNA-Seq (Westermann et al., 2012), ideally directly from in vivo samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2014.10.005.

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